Pathologic findings and causes of death in bottlenose dolphins (*Tursiops truncatus)* stranded along the Georgia Coast, USA (2007–2013)

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ABSTRACT

Between 2007 and 2013, before the 2013 cetacean morbillivirus outbreak, 26 fresh bottlenose dolphins carcasses were necropsied on the coasts of Georgia, United States. Here, we present the pathological and microbiological findings associated with their most likely causes of death. The primary cause of death was determined in 25 individuals and included systemic bacterial infection (n=7), verminous and bacterial bronchopneumonia (n=5), drowning/entanglement (n=5), disseminated histoplasmosis (n=1), intestinal intussusception (n=1), vegetative endocarditis (n=1), meningitis (n=1), necrotizing dermatitis (n=1), disseminated angiomatosis (n=1), emaciation (n=1), and stingray spine trauma (n=1). Histiocytic and eosinophilic bronchopneumonia associated with *Halocerchus* sp. infection was observed in 69% of the animals (18/26) and eosinophilic gastritis due to Anisakidae nematodes was found in 36% of the examined stomachs (8/22). Moderate to severe eosinophilic pancreatitis with fibrosis was observed in 4 animals infected with Brachycladiidae trematodes. Proliferative and ulcerative lymphoplasmacytic dermatitis was found in 5 animals and was considered to contribute to deteriorated health status in 2 calves. Pulmonary and lymph node angiomatosis were observed in 15 and 10 animals, respectively. In at least two animals, concentration of polychlorinated biphenyls (PCB) in the blubber exceeded 1,500 µg/g of lipid. Bottlenose dolphins stranded on the Georgia coast have a wide range of inflammatory lesions associated with a variety of helminth, bacterial and fungal pathogens. Some resident animals have also been exposed to high levels of PCB contamination, which could reduce host immunocompetence. Higher exposure to these or other pathogens could result in further decline in the health of resident and migrant dolphin populations in this region.

KEYWORDS: Bottlenose dolphin, Bronchopneumonia, Disease, Georgia, Marine mammals, Parasites, Pathology, PCB.

1. INTRODUCTION

Marine mammals are top predators and keystone species very sensitive to changes in ocean conditions (Simeone et al. 2015; Seguel et al. 2018; Hazen et al. 2019). Like humans and free-ranging land mammals, marine mammals are also affected by various physical and microbial agents of disease (Bossart 2011, Truchon et al. 2013), making marine mammals excellent

sentinels of ocean health (Bossart 2011). Understanding the causes and consequences of marine mammal strandings is therefore important for public, animal and ecosystem health (Simeone & Moore 2018). The study of stranded marine mammals has led to a better understanding of whale and dolphin biology, description of new species, and increased knowledge of cetacean diseases (Bogomolni et al. 2010, Arbelo 2013, Fenton et al. 2017). Understandably, many cetacean disease investigations and diagnostic efforts are pursued during mass strandings and unusual mortality events because larger numbers of fresh carcasses are available. Consequently, less is known about the baselines causes of disease and strandings in many marine mammal populations. Understanding the background health of populations is critical to accurately evaluate and interpret lesions when free-ranging animals die from emerging diseases and during epizootics, oil spills and other unusual events.

Causes of strandings are highly variable among cetaceans, and include human related causes (*e.g.*, by-catch, watercraft strikes, acoustic trauma, marine pollution), natural causes (e.g., poor nutrition, diseases), and combinations of natural and human factors (*e.g.*, harmful algal blooms) (Van Dolah et al. 2003, Simeone & Moore 2018). Among diseases, one of the most significant causes of unusual mortality events is cetacean morbillivirus (Van Bressem et al. 2014). This pathogen has caused three major epizootics in the United States in the past 35 years, resulting in the deaths of thousands of bottlenose dolphins (*Tursiops truncatus*) (Van Bressem et al. 2014). During the most recent 2013-2015 epizootic, over 1,600 bottlenose dolphins stranded dead along the Atlantic Coast of the United States (Van Bressem et al. 2014; Balmer et al. 2018), including more than 90 animals in Georgia (NOAA 2019). Although healthy dolphin populations can recover from these epizootics, cetacean populations exposed to additional health hazards, such as pollutants and/or additional infectious agents, can experience sustained declines (Van Bressem

et al. 2014). For instance, in the Caspian sea, a combination of by-catch, long-term bioaccumulation of persistent organic pollutants (POPs), and several morbillivirus epidemics led to a substantial decline of the Caspian seal (*Pusa caspica*) (Wilson et al. 2014), from which the species has not recovered (Wilson et al. 2014, Goodman & Dmitrieva 2016). This case highlights the importance of understanding how natural and anthropogenic stressors interact and affect populations *before* epizootics occur. Such information can help wildlife managers anticipate how populations may be impacted by epizootics, identify populations that are at greater conservation risk, and prioritize mitigation efforts.

Two genetically distinct morphotypes, or "stocks," of bottlenose dolphins are found in Georgia and other Southeastern U.S. coastal waters: 1) resident "estuarine" dolphins that inhabit bays, estuaries and sounds, and 2) "coastal" dolphins that inhabit nearshore Atlantic Ocean waters (Waring et al. 2016, Hayes et al. 2018). The 2013-2015 morbillivirus epizootic affected primarily coastal dolphin stocks (Morris et al. 2015), although small numbers of morbillivirus-positive animals were confirmed in several estuaries (Waring et al. 2016), including one case in Georgia (Georgia Department of Natural Resources, *unpublished data*). Transmission from coastal to estuarine stocks is not surprising given that their ranges overlap near inlets and along beaches (Waring et al. 2016, Balmer et al. 2018). However, previous live-capture/health-assessment studies in South Carolina, Georgia and Florida have found low- to nonexistent morbillivirus titers in estuarine dolphins (Bossart et al. 2010, Rowles et al. 2010, Balmer et al. 2018), suggesting estuarine stocks do not have herd immunity against morbillivirus, and could be vulnerable to morbillivirus infections when future outbreaks occur. Moreover, many estuaries are located in close proximity to human populations and are already impacted by environmental contaminants, eutrophication, habitat conversion and other human impacts (Kennish 2002). In

the Brunswick, GA area, resident estuarine dolphins have high blubber concentrations of a rare PCB mixture (Aroclor 1268) from an industrial point source. This PCB mixture has been correlated with anemia, hypothyroidism and reduced functional immune response in bottlenose dolphins (Balmer et al. 2011, Scwhacke et al. 2012). Populations with such high blubber concentrations of PCB could be especially susceptible to future morbillivirus epizootics and other emerging stressors.

In this paper we report on major pathologic findings and causes of death in bottlenose dolphins that stranded along the Georgia coast from 2007 to immediately preceding the 2013-2015 morbillivirus outbreak. Our goal is to investigate how diseases and other factors were affecting bottlenose dolphins in coastal Georgia before the epizootic occurred. We hope this baseline information will (1) provide valuable context for understanding the 2013-2015 morbillivirus epizootic, and (2) help wildlife and veterinary professionals recognize new and emerging threats that arise in the future.

2. MATERIALS AND METHODS

2.1 *Animals*

Between January 2007 and May 2013, the Georgia Department of Natural Resources (GDNR) responded to the presence of stranded (alive or dead) odontocetes along the coast of Georgia, USA. When animals were stranded alive, the National Oceanographic and Atmospheric Administration (NOAA) protocol for distressed marine mammals was applied. In cases when return to the ocean or rescue was not possible, live dolphins were euthanized according to standard protocols (Gage and Whaley 2009). All euthanized animals and carcasses in fresh

condition (code 2 and early code 3) underwent necropsy in the field or in a necropsy lab. The age class of animals (neonate, juvenile, adult) was determined based on presence of fetal folds, total body length, development of reproductive organs, and teeth wear using previously described criteria (McFee and Lipscomb 2009). All animals included in the study were considered non-infected with morbillivirus based on lack of histologic lesions indicative of the infection and negative morbillivirus immunohistochemistry of lung, respiratory lymph nodes and spleen.

2.2 *Histopathology and immunohistochemistry*

Any tissues displaying gross lesions and sections of brain, thyroid, trachea, lung, bronchial lymph nodes, heart, skeletal muscle (longissimus dorsi), kidney, adrenal gland, liver, pancreas, stomach, small and large intestine, spleen, and gonads (testis or ovaries) were routinely processed for histopathology and stained with hematoxylin and eosin (H&E) at the University of Georgia College of Veterinary Medicine, Veterinary Diagnostic and Investigational Laboratory in Athens, Georgia USA. Selected tissue sections were also stained with Gram, Grocott's methenamine silver (GMS), Warthin-Starry, Masson's trichome, Giemsa, Pricosirious red and acid-fast stains, and periodic acid-Schiff (PAS) reaction.

Sections of lung, respiratory lymph nodes, and spleen from all animals included in the study (n=26) underwent immunohistochemistry (IHC) for morbillivirus using a canine distemper virus antibody known to cross-react with cetacean morbilliviruses (Stone et al. 2011). IHC for *Histoplasma capsulatum* was performed in sections of lung, lymph nodes, and spleen from an animal with yeasts identified histologically using a commercial antibody against crude *H. capsulatum* extracts (Supplementary Table 1). Sections of adrenal gland and brain from 2 animals with adrenalitis underwent IHC against *Toxoplasma gondii* using a commercial polyclonal antibody (Supplementary Table 1). Esophageal sections with histologically identified

fungal hyphae underwent IHC for *Aspergillus spp*. fungi using a commercial antibody developed against the soluble extracts of *A. niger*, *A. flavus*, *A. fumigatus,* and *A. terreus*. The cross reactivity of this antibody with other fungal species was tested by performing the same IHC protocol in formalin-fixed sections of agar with previously identified cultures of *Alternaria* sp., *Cladosporium* sp., *Fusarium* sp., and *Microsporum canis*. Additionally, sections in agar of an *Aspergillus niger* reference strain were used as positive controls. In all IHC tests, positive controls included tissues of domestic animals from which the infectious agent was cultured, isolated and/or sequenced. In all protocols, negative controls were included by performing the same IHC procedure, except for the incubation with the primary antibody. Sections of lung and lymph nodes from 8 animals underwent K2 *Klebsiella pneumoniae* IHC as previously described in marine mammals (Seguel et al. 2017). The major details of primary antibody source, target and host species, dilution, antigen retrieval and visualization methods for all IHC protocols are provided in Supplementary Table 1.

2.3 *PCR assays*

DNA was extracted from the formalin fixed brain of 2 animals with encephalitis and from the skin of 3 animals using QIAamp DNA formalin-fixed paraffin-embedded tissues kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions but adding 2 hours of incubation of the pellet in proteinase K at 56°C. Using published primers, the *bcsp31* gene of *Brucella* spp. was amplified using real-time PCR as previously described (Probert et al. 2004). In the skin samples, 3 different PCR assays were used to investigate the presence of poxviruses. Conventional PCR assays were used to amplify low-GC and high-GC content poxviruses, as previously described (Li et al. 2010). A real-time PCR assay was used to amplify the DNA polymerase gene from *Cetaceanpoxvirus* (CePV), as previously described (Sacristan et al.

2018b). All PCRs were performed with an exogenous internal control (+IC DNA kit, High Concentration, Qiagen, Hilden, Germany) to ensure the quality of the extraction process and detect the presence of naturally occurring inhibitory compounds in the processed specimen. Synthetic DNA plasmids were used as positive controls for all PCR assays. These contained *Brucella*, low-GC or high-GC content poxviruses or CePV genes, which were inserted into plasmids pUC57 kanamycin and transformed into competent *Escherichia coli* cells (DH5α strain). Synthetic DNA plasmids were purchased from GENEWIZ (South Plainfield, NJ, USA).

2.4 *Persistent Organic Pollutants (POPs)*

Frozen blubber samples from 6 dolphins were submitted to NOAA's Center for Coastal Environmental Health and Biomolecular Research in Charleston, SC and analyzed for POPs, including 55 PCB congeners, 11 brominated diphenyl ether (BDE) congeners and a suite of organochlorine pesticides. Methods for POP extraction, quality control and calculation of total sample concentrations (in µg of POP per mg of lipid) are described in Litz et al. (2007).

2.5 *Other ancillary testing*

Metazoan parasites collected during necropsy were placed in 70% ethanol. Nematodes were cleared in lactophenol and mounted on a glass slide for morphological identification to the genus or species level following standard parasitological keys (Hartwich 1974; Delyamure 1955; Anderson 1978). Digeneans were stained in iron acetocarmine and mounted in Canada balsam for identification according to standard morphological keys (Price 1932; Delyamure 1955; Yamaguti 1971). In 4 cases, fresh tissues or swabs aseptically collected at necropsy underwent bacteriological culture using MacConkey and blood agars. Isolated bacterial colonies were

identified to the genus or species level by Gram stain and biochemical reactions using a BBL Crystal ID System for enteric/non-fermenting and gram-positive bacteria (BD, Sparks, MD).

2.6 *Immediate and contributory causes of death*

For each case, the most likely immediate cause of death was defined as the most likely disease, injury or disorder that caused the physiological derangements that directly led to the animal's death (Brownlie & Munro 2016). The subsequent contributory causes of death were those diseases, injuries, or disorders that in the context of all the postmortem findings and ancillary testing, likely accelerated or facilitated deleterious physiologic effects of the immediate cause of death.

3. RESULTS

3.1 *Causes of death*

Between 2007 and 2013, we performed necropsies and histopathology on 26 bottlenose dolphins that stranded along the Georgia coast. The details of sex, age class and the most likely cause of death of these 26 animals are summarized in Table 1. The most common primary or immediate cause of death was systemic bacterial infection (n=7), followed by drowning due to entanglement (n=5), bronchopneumonia (n=5), emaciation (n=1), disseminated histoplasmosis (n=1), vegetative endocarditis (n=1), intestinal intussusception (n=1), meningitis (n=1), necrotizing dermatitis (n=1), disseminated angiomatosis (n=1)and hemothorax due to suspected stingray spine migration (n=1) (Supplementary table 2).

3.2 *Pathologic findings and ancillary testing*

Table 2 shows registered pathologic findings in the examined dolphins. Bronchopneumonia due to *Halocercus* sp. (nematode) infection was the most prevalent condition (Fig 1A). In these animals, occasional macrophages, neutrophils and fewer eosinophils surrounded adult metastrongyles in airways and larvae in the parenchyma (Fig 1B). In 14 animals, we observed bacterial bronchopneumonia, secondary to verminous pneumonia or as a primary process. We isolated *Escherichia coli* and a B-hemolytic *Streptococcus* sp. in pure culture in two cases and one case, respectively. Interstitial pneumonia was present in cases with systemic bacterial infection and we recorded severe necrotizing interstitial pneumonia in one animal with disseminated histoplasmosis (Fig 1C). In this animal, numerous 2–4 μm diameter yeasts, admixed with cellular debris, expanded or obscured alveolar septa (Fig 1D). The yeasts were PAS-positive and stained strongly positive with anti-*Histoplasma capsulatum* polyclonal antibody (Fig 1E and 1F). In this animal, there were free and intrahistiocytic yeasts in several lymph nodes, spleen, small intestine, liver and pancreas. We recorded marked pulmonary alveolar edema in 4 dolphins that died due to drowning/entanglement.

Pulmonary angiomatosis was present in all adults and two subadult animals (total n=12). In this condition, small caliber, well-developed veins and arteries (Fig 1G), supported by mature, haphazardly arranged collagen fibers (1H, 1I), replaced large portions of the pulmonary parenchyma. In some cases, the same process occurred in lymph nodes and spleen or in most examined tissues (disseminated angiomatosis). In other cases (n=10), large amount of thin collagen fibers arranged perpendicular to small caliber blood vessels, replaced the subpleural pulmonary tissue. We considered this change consistent with pulmonary granulation tissue instead of angiomatosis.

Moderate lymphoid depletion was common in animals with a systemic or severe localized infectious process. We observed neutrophilic lymphadenitis in lymph nodes draining chronic and/or active infectious processes. In some of these cases (n=6), a similar process was observed in small areas of the splenic white pulp (mild splenitis). In the neonates with systemic gram-negative bacterial infection, there was moderate and severe splenitis. Bacteriological analysis from one of these animals yielded a pure culture of a hemolytic *E. coli.*

In the gastrointestinal system, the most common finding was ulcerative eosinophilic gastritis associated with *Anisakis* sp. and/or *Contracaecum sp*. nematode infection (n=22). In these animals, throughout the mucosa and submucosa adjacent to gastric ulcers, numerous eosinophils and fewer macrophages and multinucleated giant cells surrounded remains of nematodes cuticle (Fig 2A). In two of these animals, the pyloric submucosa contained medium size eosinophilic granulomas with sections of *Braunina* sp. trematodes in the center. We observed pancreatic fibrosis in 7 animals, and in 2 cases, fibrosis was severe and replaced most of the pancreatic exocrine and endocrine tissue with few remaining islets surrounded by occasional eosinophils (Fig. 2B). In all of these cases, there were moderate to large numbers of Brachycladiidae digenean trematodes in the pancreatic ducts. The most significant liver change was individualization and necrosis of hepatocytes in animals that died due to systemic bacterial infection (n=6). Additionally, there was marked hepatic lipidosis in 2 neonates, associated with negative energy balance based on mild wasting of skeletal muscle and thin blubber layer. We observed severe focal ulcerative stomatitis in one subadult male, probably due to trauma, and severe, focal, ulcerative, fungal esophagitis in one calf (Fig 2C). The fungal hyphae were thin, septate, with parallel walls, dichotomously branching, and stained strongly positive with anti-*Aspergillus sp*. antibodies (Fig 2D and 2E). We observed segmental, transmural, intestinal

necrosis in one case due to intussusception, in one case due to disseminated histoplasmosis, and in one case due to ulcerative, fibrinopurulent enteritis with numerous gram-negative bacilli.

In the cardiovascular system, myocardial fibrosis (n=7), associated in some cases (n=3) with enlargement, disarray and vacuolation of cardiomyocytes, was a common finding. However, all these processes were mild-to-moderate and probably did not contribute to stranding and death.

Fibrosis of the adrenal gland(s), testes, ovaries and thyroid gland was the most common finding in the endocrine tissues of adults and subadults. There were 2 cases of lymphoplasmacytic and neutrophilic adrenalitis in calves with systemic bacterial infection secondary to severe verminous and bacterial bronchopneumonia. *Toxoplasma gondii* IHC was negative in both cases.

In the skin of 3 adults and 2 neonates, we observed tattoo-target lesions in flippers, flank and abdominal midline (Fig 2F). Histologically, these lesions corresponded to proliferative and ulcerative lymphoplasmacytic dermatitis with hyperpigmentation, ballooning degeneration, and occasional intracytoplasmic amphophilic inclusion bodies (Fig 2G). We tested the neonates and one adult for poxviral infection through 3 different PCR protocols, but all assays were negative. In neonates, skin lesions covered most of the mentioned anatomical locations, and histologically there was a higher degree of ulceration and ballooning degeneration of the skin compared to the adults. Additionally, in the neonates, there were numerous filamentous bacteria, gram-negative bacilli and occasional ciliated protozoa admixed with cellular debris in the ulcerated skin and sometimes in the adjacent necrotic dermis and blubber. In these animals, we considered skin lesions to have contributed to stranding and death.

In 2 adult animals, there were extensive areas in the flank with ulcerative necrotizing dermatitis and myositis with numerous ciliated protozoa. In these cases, we considered necrotizing

dermatitis a factor that contributed to the animal stranding and death. In one sub-adult animal that died due to drowning, there was mild histiocytic and neutrophilic cellulitis associated with *Crassicauda sp*. infection.

In 2 neonates that died due to systemic bacterial infection there was mild to moderate lymphoplasmacytic and histiocytic meningoencephalitis. PCR for *Brucella sp.* and IHC for morbillivirus and *T. gondii* were negative in these cases. In 2 animals that stranded alive, there was moderate, multifocal, acute coagulative necrosis with contraction bands of longissimus muscles.

The geometric mean of pesticides, PBDE and PCB levels in the 6 animals assessed were 6.2 µg/g, 3.1 µg/g and 271.1 µg/g of lipid respectively. In 2 carcasses, PCB levels were extremely high (>1,500 µg/g lipid), including an animal that died due to disseminated angiomatosis and one that died of bronchopneumonia (Table 3). In these cases, we considered PCB exposure a contributory factor to their mortality. Both carcasses were found within 20 km of an industrial PCB point source located in Brunswick, GA, USA.

4. DISCUSSION

Determining the causes of cetacean strandings can be confounded by many factors, including low carcass detection and reporting rates, rapid decomposition of carcasses, logistical challenges of conducting necropsies in the field, and gaps in our understanding of the biology and physiology of some species. In this study, we were able to determine the likely primary and contributory causes of death for over two dozen bottlenose dolphins that stranded in Georgia over a 6-year period. This was possible thanks to a combination of (1) consistent local stranding

response and necropsy capacity, and (2) offsite diagnostic support provided by the state veterinary college and federal partners. The cases presented here are only a small fraction of bottlenose dolphins stranded in Georgia during the period (n = 145) because most carcasses were too autolyzed or decomposed to attempt diagnostics (GDNR, *unpublished data*). Nonetheless, necropsied carcasses were from a variety of age classes, they stranded during all seasons, and they were collected from a variety of habitats. Our findings should, therefore, be a reasonable proxy for morbidity and mortality that occurred in estuarine and coastal bottlenose dolphins in Georgia during the period. Within that context, infectious processes were the most common immediate or contributory cause of stranding, although drowning due to entanglement remains an important cause. This differs from other small cetacean populations where entanglement and trauma are the most common causes of stranding during years without morbillivirus epizootics or unusual mortality events (McFee and Lipscomb 2009, Fruet et al. 2012, Venn-Watson et al. 2015, Domiciano et al. 2016, Fenton et al. 2017).

Most of the pathologic processes documented in this study have been previously described in cetaceans. However, our study provides new insights into diagnostic tests and tissue reaction of bottlenose dolphins to pathogens. Additionally, the prevalence and severity of lesions due to infectious agents are higher in this study compared to other small cetacean populations in the Atlantic and Pacific oceans (McFee and Lipscomb 2009, Bogomolni et al. 2010, Fauquier et al. 2010). Among animals that died due to bronchopneumonia and systemic bacterial infection, moderate to severe verminous pneumonia due to *Halocercus sp*. infection was the most common histological finding. In bottlenose dolphins found in Florida and the Gulf of Mexico, verminous pneumonia prevalence is considerably lower than the prevalence reported in this study (Florida: 1% to 4%, Fauquier et al. 2010, Venn-Watson et al. 2015; this study: 69%), and in South

Carolina, verminous pneumonia was rarely (~4%) associated to death over a 13-year period (McFee and Lipscomb 2009). The reason for the more prevalent and severe helminth infection in Georgia is unknown, but could be related with exposure to higher number of infective stages of the parasite, and/or impaired immune function. In some marine mammal populations, inbred animals and individuals with high levels of PCBs and other pollutants have higher lungworm burdens and suffer severe verminous bronchopneumonia (Rijks et al. 2008, Jepson et al 2005), probably due to immunosuppression. Estuarine residents from the southern part of the Georgia coast have high levels of PCBs and pesticides, which has been associated with anemia, hypothyroidism, and immunosuppression (Balmer et al. 2011, Schwacke et al. 2012). We were only able to calculate blubber POP concentrations in 6 carcasses, precluding statistical association with specific lesions, but mean PCB concentrations in these 6 animals were 5-fold higher than published blubber concentrations with known reproductive and immunosuppressive effects in marine mammals (Jepson et al. 2005, Desforges et al. 2016, Murphy et al. 2018). Additionally, in 2 animals, total PCB concentrations (>1,500 ppm) are among the highest reported in any animal species (Balmer et al. 2011, Jepson et al. 2016, Murphy et al. 2018).

Pulmonary or disseminated angiomatosis has been previously described in bottlenose dolphins in the United States (Turnbull and Cowan 1999), and in other odontocete species worldwide (Diaz-Delgado et al. 2012, Domiciano et al. 2016). The etiology of this condition in cetaceans is unknown, but in humans, two similar conditions known as “cutaneous reactive angiomatosis” and “bacillary angiomatosis” are associated to vascular damage or bacterial infections that lead to tissue hypoxia and chronic inflammation respectively. These two factors could stimulate uncontrolled vascular proliferation (Resto-Ruiz et al. 2003, Rongioletti and Rebora 2003). In cetaceans, recent studies have found a correlation between the presence of angiomatosis and

lungworm infection (Diaz-Delgado et al. 2012, Domiciano et al. 2016). In this study, we did not observe a similar pattern because lungworms were present in all examined animals, and accurate estimation of parasitic burden was not attempted. However, given the marked lung parenchymal damage associated with lungworm infection in small cetaceans, it is likely that lungworms compromise the ability of dolphins to dive and/or maintain adequate oxygen exchange rates (Rijks et al. 2008, Jepson et al 2005). This could lead to tissue hypoxia and potentially stimulate blood vessel proliferation (Rongioletti and Rebora 2003).

Pulmonary edema and hemorrhage are commonly observed in cases of drowning in humans and marine mammals (Lunetta et al. 2002). However, these findings can also be observed in cases of trauma and or painful death (Lunetta et al. 2002). In our study, the diagnosis of drowning/entanglement was facilitated by the reported clinical history (animals were caught in jellyfish trawl nets or crab pot buoy lines) and the presence of fresh entanglement marks on the skin.

Lymphoid depletion was common in the studied dolphins yet was less severe than we have observed in association with morbillivirus infection (see also Van-Bressen et al. 2014). None of our cases had immunohistochemical evidence of cetacean morbillivirus infection, so that cause is very unlikely. Given that most of these animals had chronic inflammatory processes due to infectious diseases, it is possible that lymphoid depletion was due to exhaustion of the inflammatory response (Wherry 2011). Chronic PCB exposure could be another factor. Several studies of free-ranging cetaceans, including the studied population, have found poor lymphoid proliferation in animals with high levels of PCBs and pesticides (Balmer et al. 2011; Schwacke et al. 2012; Desforges et al. 2016).

We observed lymphadenitis and splenitis in cases of severe and systemic infections, mostly due to gram-negative bacteria, but also from a disseminated yeast infection (histoplasmosis). Histoplasmosis is common in domestic animals in some regions of the United States and it has been previously reported in a captive elderly bottlenose dolphin (Jensen et al. 2009). Although histoplasmosis can be a primary disease, immunosuppression favors proliferation of the yeast in multiple tissues (Kaufman 2007). Although immunosuppression could have been a contributing factor in this animal, this hypothesis is hard to confirm in a retrospective study.

Parasitic infection of the gastrointestinal system resulted in marked destruction of tissues and a strong eosinophilic inflammatory response. Gastric helminthiasis is common in fish-eating animals because intermediate stages of many helminths are contained in the viscera, fascia, and muscle of fish (Quinones et al. 2013, Romero et al. 2014). In most animal populations, these infections are associated with little or no tissue destruction and inflammation, although the burdens reported are usually low. In our study, although most animals were infected with gastric helminths, gastritis was only observed in cases where sections of anisakid nematodes were evident in histological sections, probably reflecting higher burden and/or deeper attachment of nematodes.

Eosinophilic pancreatitis and fibrosis associated with Brachycladiidae trematode infection was uncommon but usually severe. The digenean trematodes *Campula* sp. and *Brachycladium* sp. preferentially infect the hepatobiliary system and pancreatic duct, and significant inflammation and fibrosis associated with these trematodes have been reported in other cetaceans, especially in the liver (Nakagun et al. 2018). In our study, although we observed fibrosis and inflammation in the liver, the pancreas was more severely affected. In cetaceans, little is known regarding the life cycle, host species preference, and tissue tropism of these parasites, but according to the few

published studies on the subject, they can cause significant tissue damage and potentially affect the health status of dolphin populations (Jaber et al. 2004, Giorda et al. 2017).

Trauma from fishing hooks, fish bones and/or interactions with conspecifics or other cetaceans can cause oral and gastrointestinal ulcers in cetaceans (McFee and Lipscomb 2009, Venn-Watson et al. 2015, Domiciano et al. 2016). Something similar could have initially caused the focal ulcerative esophagitis we observed in one calf, however at the time of assessment there was significant inflammation and tissue damage associated with *Aspergillus sp*. overgrowth. Aspergillosis is commonly reported in captive and free-ranging bottlenose dolphins, usually in the respiratory system of immunosuppressed animals (Delaney et al. 2013, Stephens et al. 2014). In our case, aspergillosis was most likely secondary to a compromised epithelial barrier, given the localized nature of the infectious process.

Tattoo/target skin lesions are common in many odontocete populations worldwide (Van Bressem et al. 2009). One of the most common etiologies of these lesions is cetacean poxvirus-1 (Geraci et al. 1979, Sacristan et al. 2018a). Although histologic features of the tattoo lesions in this study resemble that of poxviral infection (Geraci et al. 1979, Sacristan et al. 2018a), we could not amplify poxviral DNA from formalin-fixed tissues. These negative results could ensue from excessive DNA fragmentation and cross-linking due to formalin fixation or the absence of poxviruses in the samples. Unfortunately, we were unable to perform additional ancillary testing such as electron microscopy to detect viral particles, so the etiology in our cases remains unclear. Two neonates, in addition to the proliferative tattoo-like lesions, had filamentous bacteria and ciliated protozoa in some ulcerative skin lesions. Ciliated protozoal dermatitis has been sporadically reported in bottlenose dolphins in the United States Atlantic coast (McFee and Lipscomb 2009, Schulman and Lipscomb 1999, Bossart et al. 2013), however its prevalence

increases during morbillivirus epizootics (Schulman and Lipscomb 1999), suggesting that immunosuppression could play a role in its presentation and severity.

Fibrosis in endocrine tissues, particularly in the thyroid, has been described in bottlenose dolphins, but the cause is unknown (Cowan and Tajima 2006). Some studies have related PCBs and heavy metal exposure with thyroid fibrosis in cetaceans, and an assessment of free-ranging bottlenose dolphins in Georgia found strong negative correlations between thyroid function and PCB levels (Schwacke et al. 2012). However, it is unknown if these functional abnormalities are associated with morphological changes. Adrenalitis can be caused by several infectious agents that show tropism for the adrenal gland (*e.g. Toxoplasma gondii*) or as a consequence of severe systemic viral or bacterial infections and/or sepsis (Venn-Watson et al. 2015). In our cases, the latter is the most likely explanation since all affected animals had severe gram-negative bacterial infections.

Nonsuppurative meningoencephalitis has been associated with exposure to several infectious agents and algal toxins in cetaceans (Arbelo et al. 2013, Sierra et al. 2014,Domenica Pintore et al. 2016). In this study, the cause of meningoencephalitis could not be determined and the negative results for *T. gondii* and *Brucella sp*. do not completely rule out these agents since test sensitivity can be low if performed in formalin-fixed tissues, as was the case here.

Acute necrosis with cytolysis in muscles associated with swimming has been described in live-stranded cetaceans, and is one of the hallmarks of capture myopathy in these species (Herraez et al. 2013). This finding was observed mostly in animals known to have stranded alive. Interestingly, other signs of capture myopathy, such as presence of myoglobin in renal tubules, were not observed, however more sensitive methods to detect myglobin, such as immunohistochemistry, were not performed (Herraez et al. 2013, Seguel et al. 2014).

In summary, bottlenose dolphins that stranded along the Georgia coast in years preceding the 2013-2015 morbillivirus epizootic had a high prevalence of infectious agents. Most of these pathogens caused substantial tissue damage and were contributory or primary causes of stranding and death. Additionally, some animals in this group had extremely high blubber concentrations of PCBs. Continued surveillance of environmental contaminants, infectious disease, and human impacts in Georgia's dolphins is therefore warranted. The presence of a wide range of infectious agents create a scenario in which additional threats such as environmental pollutants, fishing interactions, habitat alteration, and morbillivirus epizootics could have substantial detrimental impacts, especially in estuarine stocks with small population sizes.

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Table 1. Immediate and contributory causes of death (COD) in 26 bottlenose dolphins (*Tursiops truncatus*) found stranded along the Georgia Coast, USA between 2007 and 2013.

Case No	Sex	Age class	Immediate COD	Contributory COD 1	Contributory COD 2
1	Male	Juvenile	Bronchopneumonia		
2	Female	Adult	Vegetative Endocarditis		
3	Male	Adult	Hemothorax	Stingray spine	
4	Male	Sub-Adult	Euthanasia	Meningitis	Bronchopneumonia
5	Male	Adult	Disseminated Angiomatosis	PCB exposure	Emaciation
6	Male	Juvenile	Entanglement/Drowning	Emaciation	Bronchopneumonia
7	Male	Juvenile	Bronchopneumonia	Emaciation	Dermatitis
8	Male	Sub-Adult	Systemic Bacterial Infection	Meningitis	Ulcerative Stomatitis
9	Female	Juvenile	Entanglement/Drowning		
10	Female	Juvenile	Entanglement/Drowning		
11	Male	Neonate	Systemic Bacterial Infection	Bronchopneumonia	Enteritis
12	Female	Neonate	Systemic Bacterial Infection	Bronchopneumonia	Necrotizing Dermititis
13	Female	Juvenile	Systemic Bacterial Infection	Bronchopneumonia	Ulcerative Fungal Esophagitis
14	Female	Adult	Bronchopneumonia	Pulmonary Angiomatosis	Aging
15	Male	Adult	Disseminated Histoplasmosis		
16	Female	Adult	Necrotizing Dermatitis	Emaciation	
17	Male	Juvenile	Systemic Bacterial Infection	Emaciation	Trauma
18	Female	Juvenile	Emaciation	Bronchopneumonia	
19	Male	Adult	Systemic Bacterial Infection	Subcutaneous abscess	Emaciation
20	Male	Sub-Adult	Bronchopneumonia	Emaciation	
21	Male	Juvenile	Intestinal intussusception	SBI	
22	Male	Sub-Adult	Systemic Bacterial Infection	Bronchopneumonia	Emaciation
23	Male	Adult	Entanglement/Drowning		

24	Male	Sub-Adult	Entanglement/Drowning		
25	Female	Juvenile	Unknown	Bronchopneumonia	
26	Male	Juvenile	Bronchopneumonia	emaciation	PCB exposure

Table 2. Pathologic findings in bottlenose dolphins (*Tursiops truncatus*) stranded along the Georgia coast, USA, between 2009 and 2013.

Pathologic Finding	Severity			Total cases	Tissues examined	Prevalence (%)
	Mild	Moderate	Severe			
Whole body						
Emaciation	2	10	4	16	26	62
Respiratory System						
Verminous bronchopneumonia	6	3	9	18	26	69
Pulmonary angiomatosis	6	4	5	15	26	58
Pulmonary granulation tissue	2	5	3	10	26	38
Bacterial bronchopneumonia	3	6	5	14	26	54
Pulmonary edema	4	6	4	14	26	54
Pulmonary hemorrhage	7	0	0	7	26	27
Interstitial pneumonia	0	5	1	6	26	23
Lymphohematopoietic system						
Lymphoid depletion	2	6	3	11	24	46
Splenic EMH	5	6	0	11	24	46
Lymphoadenitis	3	4	2	9	24	38
Lymph node angiomatosis	2	4	3	9	26	35
Splenitis	3	1	1	5	24	21
Digestive System						
Verminous ulcerative eosinophilic gastritis	3	2	3	8	22	36
Pancreatic fibrosis	1	3	2	6	19	32
Portal hepatitis	3	4	0	7	26	27
Portal hepatic fibrosis	1	3	2	6	26	23
Hepatocellular necrosis	0	6	0	6	26	23
Verminous eosinophilic pancreatitis	1	1	2	4	19	21
Intestinal necrosis	0	0	3	3	21	14
Hepatic lipidosis	0	0	2	2	26	8
Ulcerative stomatitis	0	0	1	1	15	7
Ulcerative esophagitis	0	0	1	1	15	7
Cardiovascular System						
Myocardial fibrosis	5	2	0	7	22	32

Cardiomyopathy	1	2	0	3	22	14
Endocrine System						
Adrenal gland fibrosis	2	5	0	7	21	33
Adrenocortical hyperplasia	0	4	2	6	21	29
Gonad fibrosis	0	2	1	3	18	17
Adrenalitis	0	2	0	2	21	11
Thyroid fibrosis	0	0	2	2	22	9
Skin and Blubber						
Necrotizing dermititis	1	2	2	5	18	28
Dermatitis with Pox like Inclusions	0	5	0	5	18	28
Cellulitis with *Crassicauda sp.*	1	0	0	1	18	6
Central Nervous System						
Lymphoplasmacytic Meningoencephalitis	1	1	0	2	14	7
Skeletal Muscle						
Acute necrosis	0	2	0	2	21	10
Miscellaneous						
Disseminated angiomatosis	1	1	1	3	26	12

EMH: Extramedullary hematopoiesis

Table 3. Persistent organic pollutant concentrations in the blubber and most likely causes of death (COD) of 6 bottlenose dolphins (*Tursiops truncatus*) found stranded in the Georgia Coast, USA between 2009 and 2013.

Case No	Sex	Age class	ΣPCB (µg/g lipid)	ΣPest (µg/g lipid)	ΣBDE (µg/g lipid)	Immediate COD	Contributory COD 1	Contributory COD 2
5	Male	Adult	1585	16.3	5.27	Disseminated angiomatosis	PCB exposure	
8	Male	Sub-Adult	251	8.63	4.57	SBI	Meningitis	Ulcerative stomatitis
11	Male	Neonate	16.3	1.79	0.452	SBI	Bronchopneumonia	Enteritis
22	Male	Sub-Adult	400	5.29	8.25	SBI	Bronchopneumonia	Emaciation
23	Male	Adult	59.3	2.82	1.23	Drowning		
26	Male	Adult	2583	16.4	10.9	Bronchopneumonia	Emaciation	PCB exposure

PCB=polychlorinated biphenyl, Pest=Organic pesticides, BDE= brominated diphenyl ether

SBI= Systemic bacterial infection

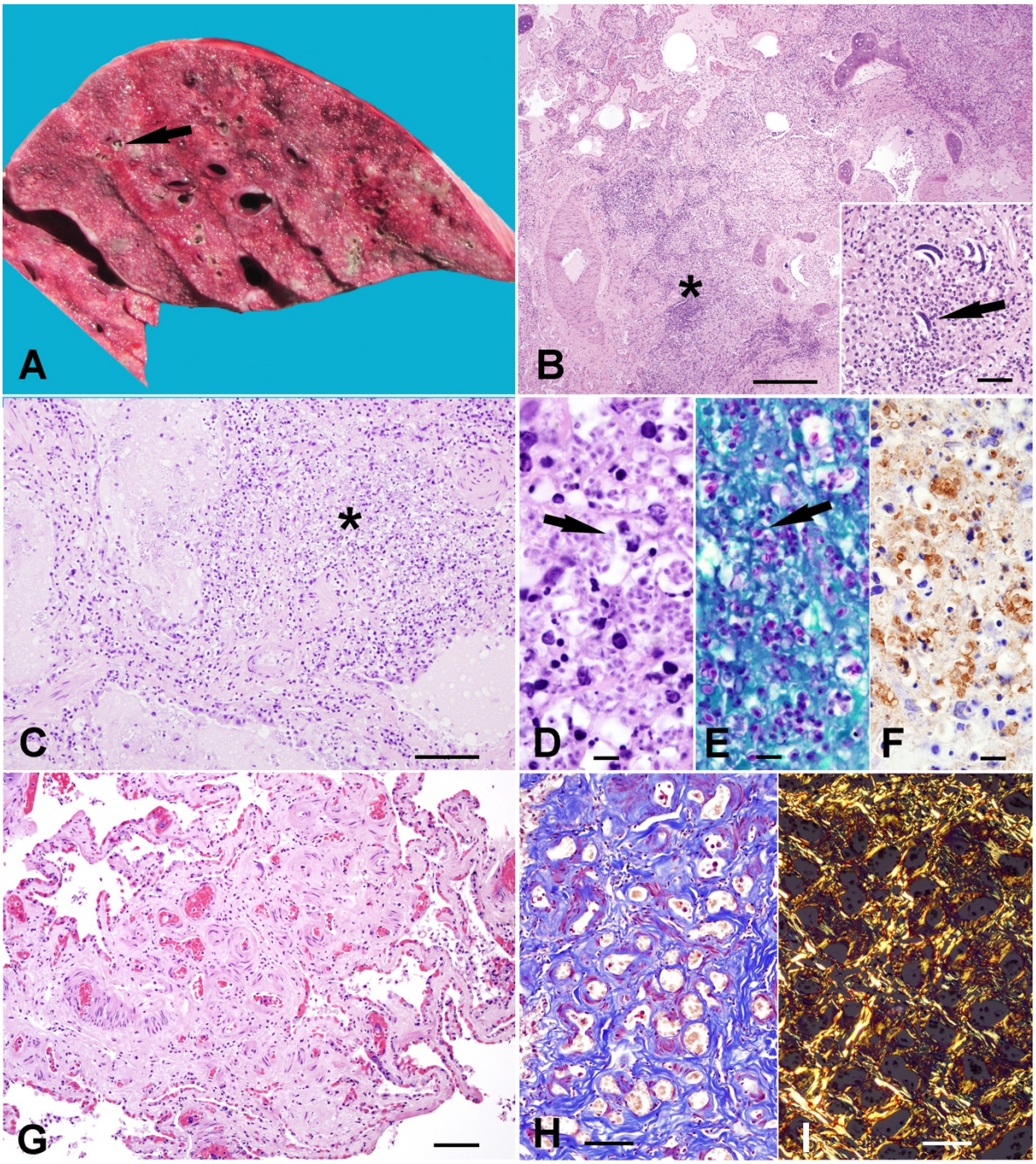

Figure 1. Respiratory system lesions in bottlenose dolphins stranded in Georgia, USA, during 2007–2013. (A) Verminous pneumonia. The lung's cut surface is diffusely dark red with multifocal areas of light brown discoloration, particularly around bronchi with nematodes (*Halocerchus spp*.) (arrow). (B) Histologic section of the lung shown in A. Inflammatory

infiltrate and cellular debris obscure the pulmonary parenchyma (asterisk). H&E; scale bar= 200 µm. Inset: Foamy macrophages and eosinophils surround metastrongyle larvae (arrow). H&E; scale bar= 20 µm. (C). Necrotizing interstitial pneumonia due to *Histoplasma sp*. infection. Cellular debris and degenerate leukocytes obscure the alveolar septum (asterisk). H&E; scale bar= 100 µm. (D) Higher magnification of lung section showed in C. Numerous intrahistiocytic and free *Histoplasma sp*. yeast surrounded by a clear halo (arrow). H&E; scale bar= 10 µm. (E) Yeasts are markedly PAS positive (purple) (arrow). PAS; scale bar= 10 µm. (F) Yeast cells have marked, diffuse, positive staining with anti-*Histoplasma capsulatum* antibodies. *Histoplasma capsulatum* immunohistochemistry with hematoxylin counterstain; scale bar= 10 µm. (G) Diffuse pulmonary neovascularization with fibrosis (angiomatosis). H&E; scale bar= 50 µm. (H) Detail of fibrous tissue in G. Fibrous tissue collagen fibers (purple) are loose and sometimes concentric in relation to blood vessels formed by prominent myocytes (red) and thin collagen fibers (purple). Masson's trichrome; scale bar= 50 µm. (I) Same section shown in H stained with picrosirius red and photographed under polarized light. Collagen fibers (yellow/red and light green) are abundant and haphazardly packed in the interstitium. Picrosirius red; scale bar= 50 µm.

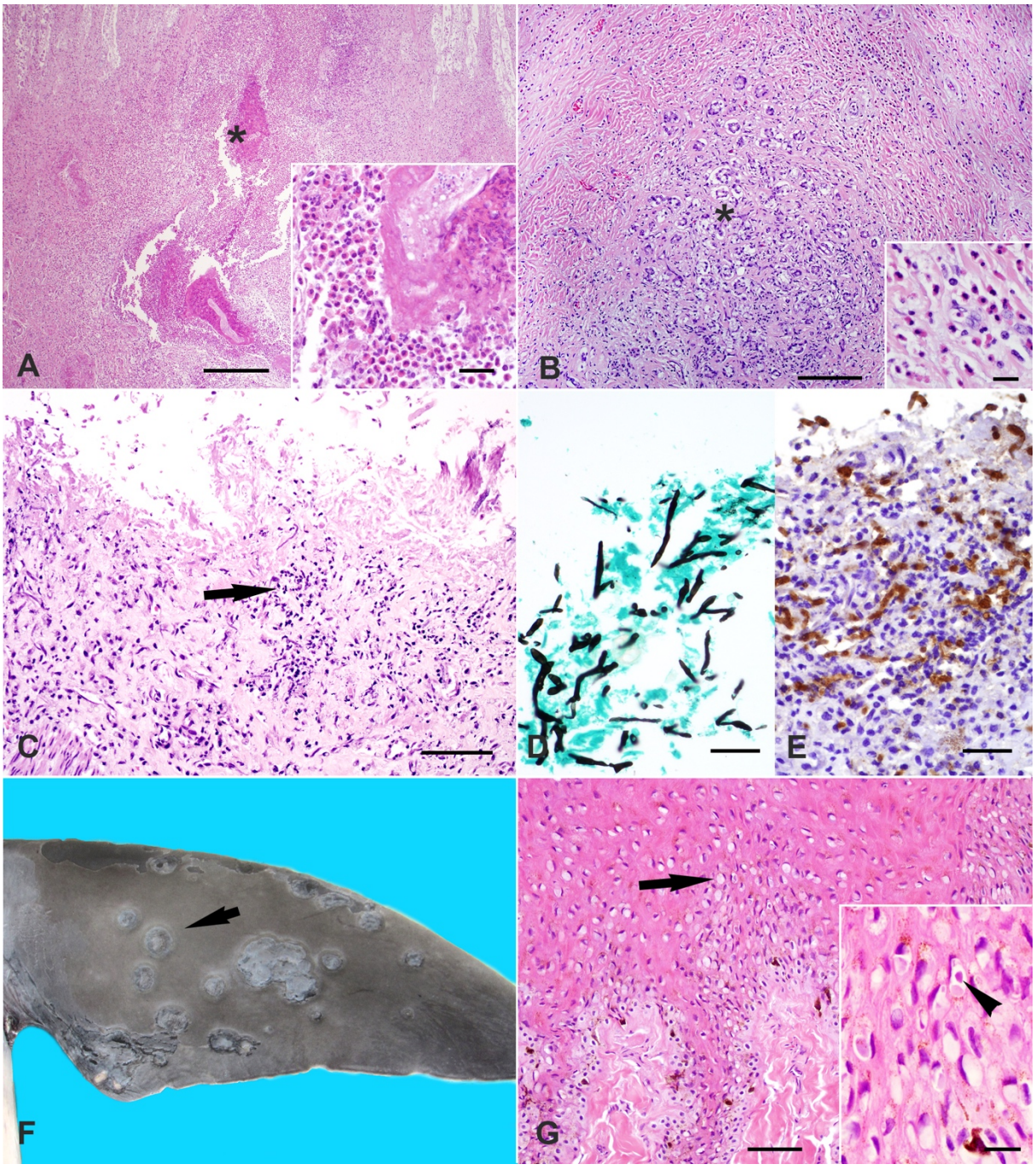

Figure 2. Pathologic findings in bottlenose dolphins stranded in Georgia, USA during 2007–2013. (A) Verminous eosinophilic gastritis. Eosinophilic necrotic debris (asterisk) obscure the submucosal histoarchitecture. H&E; scale bar= 200 µm. Inset. Numerous eosinophils surround a degenerated fragment of nematode cuticle. Scale bar= 20 µm. (B) Severe eosinophilic

pancreatitis and fibrosis. Thick collagen fibers replace and separate degenerate and atrophied exocrine pancreatic acini (asterisk). Surrounding these areas are occasional aggregates of eosinophils (arrowhead). H&E; scale bar = 100 µm. Inset. Detail of eosinophils surrounding degenerate exocrine pancreatic cells. Scale bar= 20 µm. (C), (D), and (E) Ulcerative fungal esophagitis. (C) Esophageal epithelium is lost, and in the submucosa, degenerate leukocytes surround fungal hyphae (arrow). H&E; scale bar =50 µm. (D) Fungal hyphae are septate and dichotomously branching. GMS; scale bar = 20 µm. (E) Fungal hyphae stain markedly positive with anti-*Aspergillus sp*. antibodies. *Aspergillus sp*. immunohistochemistry with hematoxylin counter-staining; scale bar = 20 µm. (F) and (G) Multifocal to coalescing hyperplastic dermatitis. (F) Tattoo/target lesions (arrow) coalesce in a pectoral flipper. (G) There is hyperplasia, ballooning degeneration (arrow) and occasional melanomacrophages in the epidermis. H&E; scale bar = 50 µm. Inset. Detail of degenerate lipokeratinocytes with peripheralized nucleus and intracytoplasmic amphophilic inclusion bodies (arrow heads). H&E; scale bar = 10 µm.

Supplementary table 1. Details of primary antibody source, type, host species, antigen retrieval and visualization methods in immunohistochemical protocols used in the study.

Antibody	Source (company)	Antibody type, host species, antigen	Antigen Retrieval Method	Primary Antibody dilution	Visualization Method
Histoplasma capsulatum	Gibson Labs[a]	Polyclonal, rabbit, Anti-*H. capsulatum*	None	1:10,000	DAB
Toxoplasma gondii	VMRD[b]	Polyclonal, goat, Anti-*T. gondii*	Protease 3	1:10,000	DAB
Canine Distemper Virus	VMRD	Monoclonal, mouse, Anti-CDV nucleoprotein	Citrate	1:1500	DAB
Aspergillus sp.	Abcam[c]	Polyclonal, rabbit, Anti-*Aspergillus sp.*	Citrate	1:10,000	DAB
K2 Klebsiella pneumoniae	SSI[d]	Polyclocal, rabbit, Anti-K2 capsular Ant.	None	1:1000	DAB

[a]Gibson Bioscience, Lexington, KY, USA.

[b]Veterinary Medical Research and Development, Pullman, WA, USA

[c]Abcam (ref. number ab20419), San Francisco, CA, USA.

[d]Staten Serum Institut, Copenhagen, Denmark.

Supplementary table 2. Summary of the immediate cause of death (COD) by sex and age class in bottlenose dolphins (*Tursiops truncatus*) stranded along the coast of Georgia, USA, between 2007-2013.

Immediate COD	sex		age				Total number cases
	male	female	neonate	juvenile	sub-adult	adult	
Systemic bacterial infection	5	2	2	2	2	1	7
Bronchopneumonia	4	1	0	3	1	1	5
Entanglement/Drowning	3	2	0	3	1	1	5
Vegetative endocarditis	0	1	0	0	0	1	1
Hemothorax	1	0	0	0	0	1	1
Euthanasia	1	0	0	0	1	0	1
Disseminated angiomatosis	1	0	0	0	0	1	1
Disseminated histoplasmosis	1	0	0	0	0	1	1
Necrotizing dermatitis	0	1	0	0	0	1	1
Emaciation	0	1	0	1	0	0	1
Intestinal intussusception	1	0	0	1	0	0	1
Unknown	0	1	0	1	0	0	1